

# Uptake of Copper Complexed to EDTA, Diaminoethane, Oxalic Acid, or Tartaric Acid by Neon Tetras (*Paracheirodon innesi*)<sup>1</sup>

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**Uptake of copper complexes by fish was studied by exposing neon tetras (*Paracheirodon innesi*) to solutions containing different concentrations of copper complexed to EDTA, diaminoethane, oxalic acid, or tartaric acid, while other copper species were kept at low and constant levels. The distribution of the copper species in the solution was quantified using chemical equilibrium modeling. It was found that the presence of the organic ligands studied could reduce, but not totally eliminate, the uptake of copper by fish. The rate of the additional uptake besides the contribution of free copper depended on the conditional stability constant of the copper complexes.** © 2002 Elsevier

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**Key Words:** bioavailability; fish; copper; copper complex.

## INTRODUCTION

It is generally accepted that the chemical form of a metal in an aquatic system is essential with respect to its bioavailability and/or toxicity. However, knowledge of the relationship between specific metal species and their availability to various aquatic organisms, as well as the processes and physiochemical factors governing the uptake and accumulation of particular metal species, is not sufficient (Hudson, 1998).

The actual binding to naturally occurring organic matter and resulting sorption onto suspended sediments are of particular importance to metal speciation and activity in aquatic environments. An investigation into the content and migration forms of a number of heavy metals in water from two reservoirs indicated that the binding of heavy metals in complexes with dissolved organic matter or their adsorption onto suspended particles was the main reason for low levels of free ions (Linnik, 1996). Both organic ligands and sus-

pending particles compete with inorganic ligands and the functional groups found on biological surfaces for metal binding and, consequently, influence metal bioavailability (Allen *et al.*, 1980). With respect to dissolved species, it has been reported in the literature that the presence of naturally occurring organic compounds may reduce metal uptake by aquatic organisms through complexation (Hollis *et al.*, 1996). However, a limited number of papers claimed that metal accumulation is enhanced by the presence of naturally occurring organic material (Giesy *et al.*, 1977; Benson *et al.*, 1994). Others claimed that there was an apparent accumulation of fulvic acid-bound copper in fish gills (Tao *et al.*, 1999a).

The possible bioaccumulation of metal–organic ligand complexes by fish gills is similar to that which occurs when metal is sorbed onto the particles. Neither is able to penetrate directly across the epithelial cell membrane of the fish gills. The uptake of these species, if possible, has to occur through a process of transformation. Recently, it was reported that the copper sorbed onto kaolin clay was available for uptake into fish via the gills (Tao *et al.*, 1999a). As a result, a mechanism for particulate metal uptake by the gills was suggested. The essential point of the proposed uptake mechanism was the desorption of the metal from the particles within the gill microenvironment in the area where the particles adhered to mucus (Tao *et al.*, 1999b).

It is speculated that copper complexed to organic ligands may undergo a dissociation process in the fish gill microenvironment similar to those sorbed onto particles. To verify this speculation, a set of exposure experiments was designed using modeled organic ligands.

## MATERIALS AND METHODS

The exposure experiments were conducted in a solution synthesized by adding reagent-grade salts to deionized water. The ionic composition of the major rivers of China was adopted as the basis for the synthetic water used in this study. The major cation and anion concentrations of the

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water were  $[\text{Na}^+]$  0.144 mmol/L,  $[\text{Ca}^{2+}]$  0.710 mmol/L,  $[\text{K}^+]$  0.009 mmol/L,  $[\text{Mg}^{2+}]$  0.324 mmol/L,  $[\text{Cl}^-]$  1.733 mmol/L, and  $[\text{SO}_4^{2-}]$  0.324 mmol/L, respectively. pH ranged between 7.2 and 7.3. Neon tetras (*Paracheirodon innesi*) approximately 15 mm in size were obtained from a fish market in Beijing. Once in the laboratory, the fish were placed in the synthetic water solution for 5–7 days as an acclimation period, prior to use in the exposure experiments.

Four ligands—EDTA, tartaric acid, oxalic acid, and diaminoethane—were selected for the study; these have conditional stability constants of complexation for copper of 18.70, 6.51, 8.50, and 10.45 log units, respectively. Exposures were initiated by transferring eight fish to each of 24 separate glass tanks each holding 800 mL of synthetic water. The water was spiked with copper and one of the four ligands at various concentrations. The concentrations of the spiked ligands were calculated using the conditional stability constants of the complexes and the designated levels of complexed copper (Table 1). When the concentration of complexed copper increased from 0 to 20  $\mu\text{mol/L}$ , the non-organic ligand-complexed copper maintained a relatively low and constant level of 1.0  $\mu\text{mol/L}$ , with exception of the control group (No. 0).

The exposure experiments lasted 6 days. A number of parameters including temperature, pH, inorganic carbon, total organic carbon (TOC), alkalinity, and UV absorbance at 190 nm, were measured at the beginning of the experiment (Day 0) and each day afterward (Days 1–6). For the EDTA system, more samples were collected during the first 2 days (Days 0, 0.2, 0.9, 1.2, 2–6). The data collected during daily monitoring were used to assess the possible changes in copper speciation that might occur during the exposure period. The fish were not fed during their exposure, avoiding the potential for a significant change in metal speciation. The water was continuously aerated with an air pump.

After exposure, the fish from each tank were randomly pooled into two groups of four individuals for a duplicate copper analysis. The samples were weighed and digested using a CEM microwave oven (Model MDS-2000). Concentrated nitric acid (5.0 mL) and 30% hydrogen peroxide (2.0 mL) were used for digestion in a stepwise mode. The resulting residue was then evaporated on a hot plate (200°C) and totally dissolved in deionized water such that no solid residue was left. The solution was neutralized using 1.0 mol/L NaOH and brought to 50 mL for copper measurement.

Copper content was measured using a PAR polarograph (Model 384) in a differential pulse anodic stripping mode, in conjunction with a Model 303 hanging mercury drop electrode. The sample solution was buffered with 1.0 M NaAc-HAc (1 mL buffer and 9 mL sample) and was purged for 10 min. A deposition time of 120 s, equilibrium time of 30 s, scan range of 0.5–0.1 V, voltage pulse height

**TABLE 1**  
Setup of the Exposure Experiments

	Group					
	0	1	2	3	4	5
Total Cu ( $\mu\text{mol/L}$ )	0.0	1.0	2.0	6.0	11.0	21.0
Complexed Cu ( $\mu\text{mol/L}$ )	0.0	0.0	1.0	5.0	10.0	20.0

of 50 mV, and scan rate of 4 mV/s were adopted for the study.

A single-site complexation model was used to describe the interaction between copper and mucus as

$$k = \text{ML}/[M_f(L_t - \text{ML})], \quad (1)$$

where  $k$  is the conditional complexation stability constant, ML (mol/L) represents the concentration of the copper–mucus complex, and  $M_f$  (mol/L) and  $L_t$  (mol/L) are the concentrations of free metal and total mucus, with the latter representing the normal concentration equivalent of complexed copper.  $L_t$  represents the product of the mass concentration of mucus in organic carbon (TOC, mg C/L) and the complexation equivalent concentration of unit mucus ( $L_0$ , mol Cu/mg C). The complexation capacity of the fish gill mucus was titrated with copper using a copper ion-selective electrode (Model Pcu-1,  $10^{-7}$ –0.1 mol/L), a reference electrode, and a PHS-2 pH meter. The electrode was activated in  $\text{Cu}(\text{NO}_3)_2$  (1 mol/L) for 2 h and rinsed with deionized water before use. The titration was carried out in the synthetic water.

The activity of free copper ions at each calibration point was calculated using the MINTEQA2 speciation model based on the total copper concentration together with other parameters, such as pH, alkalinity, and major ion concentrations as inputs to the model. The calculated mucus–copper complexation stability constant was 5.77 (log units), which is very close to the 5.37 derived using an *in situ* measurement and iterative calculation process for carp (*Cyprinus carpio*) (Tao *et al.*, 2000b).

The alkalinity of the samples was titrated with a calibrated HCl solution. pH was determined using a PHS-2 pH meter coupled with an F-13 pH combination electrode calibrated in standard solutions of pH at 6.86 and 9.18. A Shimadzu 5000A TOC analyzer was used for measurement of the total inorganic (IC) and organic (TOC) carbon. The TOC analyzer had a detection limit below 0.2 mg C/L. The absorbance of the solution at 190 nm was recorded using a Unicam UV4-100 UV/VIS spectrometer. All of the reagents employed in the experiment were of analytical grade or better. Deionized water was used throughout for copper determination. All glassware was soaked in 10% nitric acid (v/v) for 24 h and rinsed with deionized water before use.

## RESULTS AND DISCUSSION

## Copper Speciation in the Solution

The distribution of copper species in the exposure system was carefully designed such that the uptake of noncomplexed copper by fish would remain constant, allowing assessment of the contribution of the organic ligand-complexed copper on fish accumulation. However, excretion of mucus from the fish and unexpected changes in pH and alkalinity of the water during the exposure period could cause a redistribution of copper species. To evaluate the possible influence such changes might have on the experiment, copper speciation in the water was calculated based on daily measurements of the various parameters. Figure 1 illustrates the copper speciation in the EDTA-Cu system (the designated concentrations of total and EDTA-complexed copper were 6.0 and 5.0  $\mu\text{mol/L}$ , respectively) on the first day of the experiment. Only the calculated dominant copper species are presented, accounting for 99.7% of the total copper in solution.

The other dominant species were  $\text{Cu}(\text{OH})_2$ ,  $\text{CuCO}_3$ , mucus-Cu complex, and  $\text{Cu}^{2+}$ . The results in Fig. 1 are typical of the chemical equilibrium calculations for all of the ligands and for all days. Figures 2 and 3 illustrate the calculated levels of EDTA-Cu and other dominant copper species in five sets of the exposure solution. The concentrations of EDTA-Cu were almost identical to the designated concentrations, while the other species remain constant as required. Two conclusions can be drawn from these results. First, the actual concentrations of copper complexed with EDTA, diaminoethane, tartaric acid, or oxalic acid in the solution were very close to the expected concentrations. Second, not only the concentration, but also the species distribution among other dominant species remained more or less constant.

The changes taking place in the various parameters over time were also investigated. For all four of the ligands at various concentrations, the results were more or less the same. One representative sample, that of tartaric acid (1  $\mu\text{mol/L}$ ), is presented in Fig. 4.

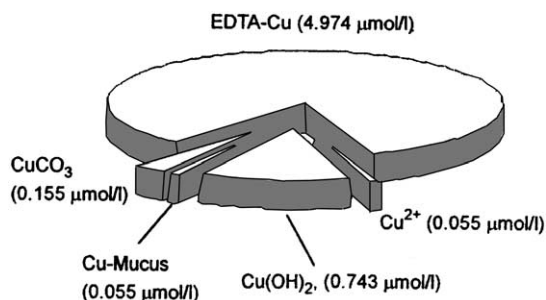


FIG. 1. Distribution of dominant copper species in the exposure solution (designed total Cu: 6.0  $\mu\text{mol/L}$ , EDTA-Cu: 0.50  $\mu\text{mol/L}$ ).

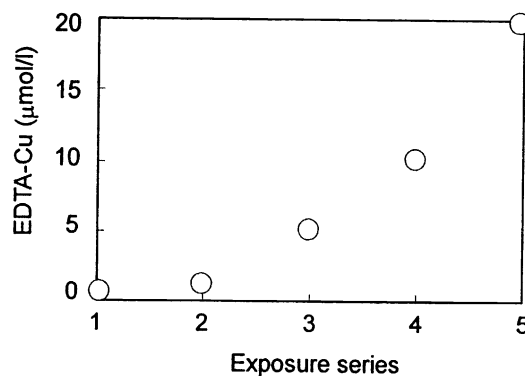


FIG. 2. Calculated EDTA-Cu concentrations in the water with designed EDTA-Cu values of 0, 1, 5, 10, and 20  $\mu\text{mol/L}$ , respectively.

As can be seen in Fig. 4, the parameters change very little over the course of the 6-day exposure period with the exception of mucus content, which increased significantly. Copper speciation, however, did not change much due to the relatively low conditional complexation stability constant for mucus-Cu ( $\log k = 5.77$ ). Accordingly, no significant changes in major copper species were observed during the experiment. Therefore, only the mean values of the daily measurements were calculated and used in this study.

## Copper Accumulation in Fish as a Function of Complexed Copper in the Solution

As indicated in Figs. 2 and 3, the exposure concentrations of copper when complexed with EDTA, diaminoethane, tartaric acid, or oxalic acid increased from 0 to 20  $\mu\text{mol/L}$ , while the other copper species remained at relatively low and constant levels (1.0  $\mu\text{mol/L}$  in total). These results indicate that the copper accumulated in the fish apparently increased with increases in the level of exposure to the various complexes (Fig. 5).

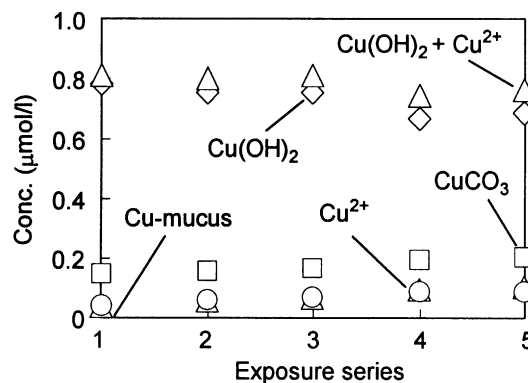


FIG. 3. Calculated concentrations of dominant copper species (except EDTA-Cu) in the water with designed EDTA-Cu values of 0, 1, 5, 10, and 20  $\mu\text{mol/L}$ , respectively.

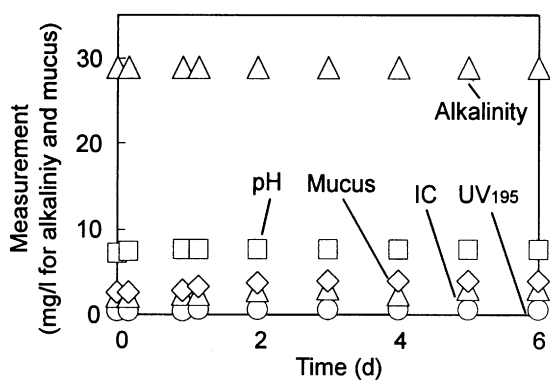


FIG. 4. Change in alkalinity (in  $\text{CaCO}_3$ ), pH, mucus (in total carbon), IC, and  $\text{UV}_{195}$  absorbance during the 6 days of the exposure experiment.

Fish exposed to the synthetic solution without copper or ligand spiking were used as the control group (No. 0 in Table 1). Copper concentrations in these fish were subtracted from the results presented in Fig. 5, and thus the results represents the “pure” accumulation associated with the exposure period. The results indicate that for all four ligands studied, the amount of copper accumulated in the fish was positively proportional to the complexed copper concentration exposure. Because the background copper concentration in the fish was taken into account by means of a control level subtraction, a simple linear forced to zero seems to fit the data quite well. The derivation of regression equations as a result of this process and their associated  $R^2$  values are as follows:

$$[\text{Cu}]_f (\mu\text{g/g wet wt}) = 0.545 [\text{Cu-EDTA}]_w (\mu\text{mol/L}),$$

$$R^2 = 0.948,$$

$$[\text{Cu}]_f (\mu\text{g/g wet wt}) = 0.775$$

$$[\text{Cu-diaminoethane}]_w (\mu\text{mol/L}), \quad R^2 = 0.974,$$

$$[\text{Cu}]_f (\mu\text{g/g wet wt}) = 1.042$$

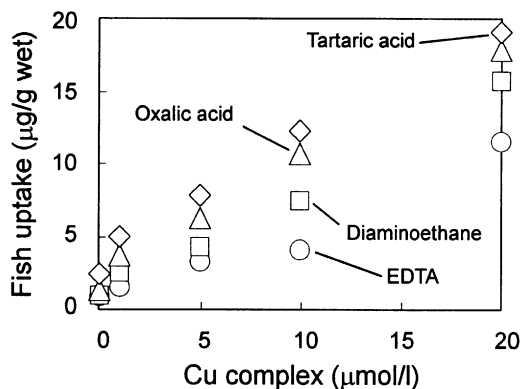


FIG. 5. Increase in fish uptake of copper as a function of the concentration of the complex.

$$[\text{Cu-tartaric acid}]_w (\mu\text{mol/L}), \quad R^2 = 0.812,$$

$$[\text{Cu}]_f (\mu\text{g/g wet wt}) = 0.948$$

$$[\text{Cu-oxalic acid}]_w (\mu\text{mol/L}), \quad R^2 = 0.921.$$

$[\text{Cu}]_f$  represents the copper accumulated in fish,  $[\text{Cu-ligand}]_w$  is the exposure concentration of the copper complex in solution, and  $R^2$  is the coefficient of determination for the regression analysis.

There is a body of literature that implies that it is the free metal ion that controls bioavailability, while the soluble metal complexes remain nonbioavailable (Hollis *et al.*, 1996; Linnik, 1996; Welsh *et al.*, 1996). For example, it has been reported that naturally occurring dissolved organic matter (DOM) kept copper from accumulating in small rainbow trout (*Oncorhynchus mykiss*) during a 9-day exposure to  $0.5 \mu\text{mol/L}$  copper. This seems to suggest that the protective effects of DOM are a result of the formation of Cu-DOM complexes, which reduce the amount of free copper in the water (Hollis *et al.*, 1997). Further, it would seem to imply that the Cu-DOM complexes themselves do not bind to the gills (Hollis *et al.*, 1997). However, in addition to evidence suggesting that hydroxy complexes of some metals are bioavailable to fish (Cowan *et al.*, 1986; Tao *et al.*, 2000a), there are other findings demonstrating the accumulation of metals bound to natural occurring organic material in fish (Giesy *et al.*, 1977; Tao *et al.*, 1999a).

The results presented in Fig. 5 indicate that copper complexed with EDTA, or the other organic ligands studied, contributed in a meaningful way to copper accumulation in fish. To compare the bioavailability of both complexed copper and free ions, an “accumulative” ratio was developed. It is defined as the ratio of the accumulated copper concentration in the fish ( $\mu\text{mol/g}$ ) to the copper concentration in the water ( $\mu\text{mol/mL}$ ) to which the fish were exposed. The accumulative ratio 15 expressed in milliliters per gram ( $\text{mL/g}$ ). Based on the regression equations presented above, the accumulative ratios for fish exposed to copper complexed with EDTA, diaminoethane, tartaric acid, or oxalic acid for 6 days were 8.58, 12.2, 16.4, and 14.9  $\text{mL/g}$ , respectively, with a mean value of  $13.0 \text{ mL/g}$ . Using the same definition, and based on results from the zero exposure period (No. 1 in Table 1), the accumulative ratio for fish exposed to copper species other than those complexes was also calculated. The resulting ratio was  $20.6 \text{ mL/g}$ . If, however, only the free cupric ions and their hydroxy complexes are considered bioavailable and copper complexed by carbonate or mucus could not be absorbed, the ratio increases to  $47.1 \text{ mL/g}$ . Regardless of the means of calculation, the accumulation ratios for the organic ligand-complexed copper are smaller than those for other species. This would seem to indicate that these ligands may provide some sort of inhibiting effect on fish accumulation. Indeed, this effect has often been taken as evidence supporting the concept that

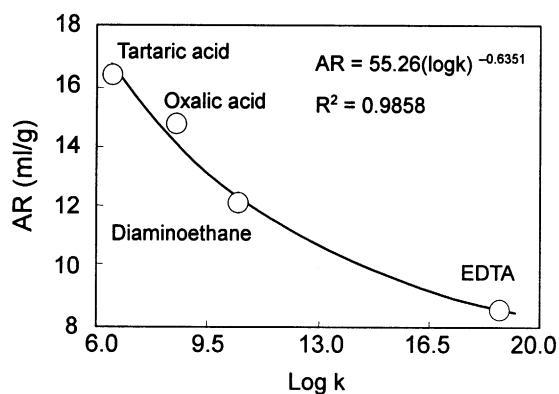


FIG. 6. Relationship between the accumulative ratio (AR) and the conditional complexation stability constant ( $\log k$ ).

metal complexes are nonbioavailable. The stoichiometric evidence provided by this study demonstrates that copper complexed by organic ligands such as EDTA is apparently available for fish uptake, but to a much lesser extent compared with free ions or hydroxy complexes.

#### *Differences among the Metals Complexed with the Various Ligands Studied*

Theoretically, the copper complexed with the four organic ligands presented in this study is not able to transfer directly across the epithelial cell membrane of fish gills. Uptake at the fish gill has to occur indirectly, and a transformation of the complexes to other readily available forms must occur. This kinetic dissociation of the complexes could take place within the fish gill microenvironment near the gill surface, where the pH is generally lower and the ionic strength normally higher than in the bulk solution (Miller and Mackay, 1982; Lin and Randall, 1990). In a similar study dealing with the bioavailability of particulate copper, it was found that the copper sorbed onto particles was able to accumulate in fish gills (Tao *et al.*, 1999a). This research suggested that the particles adhered to the epithelial cell surface, with the desorption of copper occurring before the particles were sloughed off the gill surface (Tao *et al.*, 1999b). A similar process might be occurring in copper complexed with EDTA, diaminoethane, oxalic acid, or tartaric acid. Results of the present research seem to suggest that the complexes may be adhering to the mucus layer on the epithelial cell surface during fish aspiration. Dissociation of the complex could then release free copper, which, in turn, translocates into the gill tissue. The free ligands themselves are stripped off together with the fish gill mucus that is continuously being replaced.

If the suggested uptake mechanism for the copper complexes could be somehow corrected, then the accumulation of the copper complexed with the various ligands would vary depending only on the strength of the complexation. This correction can be accomplished by plotting the accu-

mulative ratios (ARs) for the four copper complexes studied against their conditional complexation stability constants in log units. This has been done in Fig. 6.

As expected, for the four copper complexes studied, the more stable the complex, the less uptake by the fish. The form of the relationship between the accumulative ratio and stability constant is that of a power function. The coefficient of determination for the equation was very close to 1 (0.9858). This strong binding evidenced between copper and EDTA, compared with other ligands, clearly restricts the dissociation of the copper complex in the fish gills.

#### *Differences in Mucus Secretion*

As indicated in Fig. 4, among the major parameters measured, the amount of mucus in the exposure solution was the only parameter that increased significantly during the experimental period (due to fish secretion). The increase occurred in all tanks, including those not spiked with copper. However, it is interesting to note that the mucus increased even faster as the amount of complexed copper in the system increased. See the example in Fig. 7 (EDTA-Cu system).

For the tank without EDTA-Cu, the amount of mucus in solution increased gradually during the first 2 days, remaining constant thereafter. The addition of EDTA-Cu to the solution caused more mucus to accumulate and the increasing trend to last longer. Varanasi and Markey (1978) found that the amount of mucus secreted from the gills of *Oncorhynchus kisutch* increased significantly when the fish were exposed to either lead or cadmium. Similar phenomena have been observed in a number of other species and for a variety of metals (Eddy and Fraser, 1982; Randall *et al.*, 1991).

#### CONCLUSION

It is believed that the accumulation of copper in fish gills stimulates the secretion of gill mucus. The increased levels of

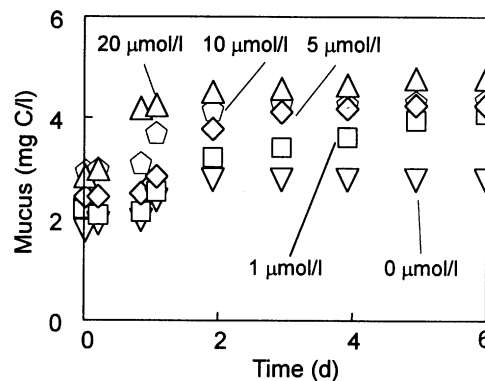


FIG. 7. Change in mucus concentration in water at various EDTA-Cu exposure concentrations during the 6-day exposure experiment.

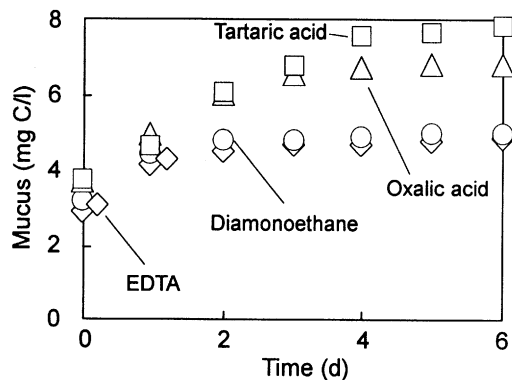


FIG. 8. Change in mucus concentration in the water with different copper complexes at 20 μmol/L during the 6 days of the experiment.

mucus in solution (Fig. 7) serve as further evidence supporting the fish gill uptake of copper complexes (all other copper species remained constant). In fact, mucus content was a function not only of the copper complex dosage, but also of the type of ligand in the solution (Fig. 8).

The influence of the copper complexes on mucus secretion follows exactly the same relational order as does fish uptake (Fig. 5) and the conditional complexation stability constant. The results seem to add further support to the contention that increased accumulations of copper in the fish are associated with increased mucus secretion.

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